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(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).

(72) Inventors: JESSEE, Joel, A.; 4139 Old National Pike, Mount Airy, MD 21771 (US). BLOOM, Frederic, R.; 19456 Caravan Drive, Germantown, MD 20874 (US). TRINH, Thuan; 209 Tulip Drive, Gaithersburg, MD 20877 (US).

(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

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(54) Title: METHODS FOR LYOPHILIZING COMPETENT CELLS

(57) Abstract

This invention relates to a method for producing cells which are competent for transformation and which may be stably stored for extended periods of time at various temperatures. The method involves growing cells in a growth conducive medium, rendering said cells competent, and lyophilizing said competent cells. The invention further relates to competent cells produced by such a method, to methods of transforming said cells with a DNA molecule, and to a method of producing a desired protein or polypeptide from said transformed cells.

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Methods for Lyophilizing Competent Cells

Field of the Invention

This invention relates to a method for producing cells which are competent for transformation and which may be stably stored for extended periods of time at various temperatures. The method involves growing cells in a growth conducive medium, rendering said cells competent, and lyophilizing said competent cells. The invention further relates to competent cells produced by such a method, to methods of transforming said cells with a DNA molecule, and to a method of producing a desired protein or polypeptide from said transformed cells.

Background of the Invention

A number of procedures exist for the preparation of competent cells and the introduction of DNA into these cells. For example Mandel and Higa (*Journal of Molecular Biology 53*:159 (1970)) describe a procedure whereby bacteriophage DNA is combined with *E. coli* cells in the presence of 50 mM Ca^{++} at 0°C, followed by a brief heat pulse at 37°C- 42°C. This method has been extended to the uptake of chromosomal DNA (Cosloy and Oishi, *Proceedings of the National Academy of Science 70*:84 (1973)) and plasmid DNA (Cohen *et al.*, *Proc. Natl. Acad. Sci. USA 69*:2110 (1972)). A summary of the factors influencing the efficiency of transformation is given in Hanahan (*JMB 166*:557 (1983)). These factors include the addition of other cations such as Mg, Mn, or Rb to the Ca-treated cells as well as the prolonged incubation of the cells in $CaCl_2$. The efficiency of transformation of *E. coli* cells is substantially enhanced by the method described by Hanahan (JMB (1983), hereinafter referred to as "Hanahan (1983)"). In the Hanahan (1983) method, the cells are grown at 37°C in the presence of 20 mM Mg. Plasmid DNA is combined with the cells at 0°C in the presence of Mn, Ca, Rb or K, dimethylsulfoxide (DMSO), dithiothreitol (DTT) and hexamine cobalt chloride. Competent cells of several strains of *Escherichia*

coli (*E. coli*) prepared by the latter method have transformation efficiencies of from 1 to 5 x 10^8 transformants/μg plasmid DNA.

Another method for the preparation of competent *E. coli* cells is disclosed in U.S. Patent No. 4,981,797 (Jessee and Bloom, 1991). That method includes the steps of growing the cells in a growth-conducive medium at a temperature of less than 37°C, rendering the cells competent and then freezing them.

Generally, frozen competent cells prepared by methods such as those summarized above have transformation efficiencies of about 1 x 10^8 transformants/μg plasmid DNA. These competent cells can be stored at -80°C for several months without significant loss of transformation efficiency. However, cells prepared by the methods outlined above are extremely unstable when stored at temperatures higher than -80°C (*e.g.*, -20°C). Stable storage of competent cells at higher temperatures is highly desirable, since many research labs do not have access to -80°C freezers.

Summary of the Invention

The present invention relates to a method which allows competent cells to be lyophilized and stored for extended periods of time at various temperatures (-80°C to room temperature) without appreciably losing transformation efficiency. Thus, the method of the invention provides competent cells which do not require specialized storage conditions (*e.g.*, extremely low temperatures) to maintain the transformation efficiency of such cells.

The method of the invention comprises growing the cells in a growth conducive medium, rendering said cells competent, and lyophilizing said competent cells. According to the method of the invention, the cells are rendered competent by incubating the cells in a competence buffer, preferably at a low temperature (*e.g.*, 0°C to 4°C). The cells are then lyophilized in the presence of a cryoprotectant.

The invention further relates to competent cells produced by such a method. In a preferred embodiment, the cells are *Escherichia* cells, most preferably *E. coli*. In a more preferred embodiment, the *E. coli* cells are selected from the group consisting of DH5α and DH10B.

In another embodiment, the invention relates to a method for transforming the lyophilized competent cells produced by the above-summarized method with a DNA molecule. The invention also relates to the transformed cell produced by this method.

In another embodiment, the invention relates to a method of producing a desired protein by first obtaining a lyophilized competent cell produced by the above method, transforming the lyophilized competent cell with a DNA molecule capable of expressing the desired protein, and then culturing the transformed cell under conditions sufficient to produced the desired protein. The invention also relates to a protein produced by this method.

Brief Description of the Figures

Figure 1 is a graph showing the transformation efficiency of competent *E. coli* DH5α that had been lyophilized and stored at -20°C in NUNC cryovials, where the cryoprotectant used was either trehalose or sucrose.

Figure 2 is a graph showing the cell viability of competent *E. coli* DH5α that had been lyophilized and stored at -20°C in NUNC cryovials, where the cryoprotectant used was either trehalose or sucrose.

Figure 3 is a graph showing the transformation efficiency of competent *E. coli* DH5α that had been lyophilized and stored at -20°C, where the cryoprotectant was trehalose, and the product was vacuum sealed in glass vials.

Figure 4A is a graph showing the transformation efficiency of competent *E. coli* DH5α that had been lyophilized in NUNC cryovials, where the cells were first frozen at -80°C for 16 hours, lyophilized, and then stored at -20°C for varying periods of time.

Figure 4B is a graph showing the transformation efficiency of competent *E. coli* DH5α that had been lyophilized in NUNC cryovials, where the cells were first frozen in liquid nitrogen, lyophilized, and then stored at -20°C for varying periods of time.

Figure 5 is a graph showing the cell viability of competent *E. coli* DH5α that had been lyophilized in NUNC cryovials and stored at -20°C for varying periods of time, where lyophilization and storage at -20°C is preceded by either freezing at -80°C for 16 hours, or freezing in liquid nitrogen.

Figure 6 is a graph showing transformation efficiency of competent *E. coli* DH5α that had been lyophilized according to Example 3.

Figure 7 is a graph showing the cell viability of competent *E. coli* DH5α that had been lyophilized according to Example 3.

Figure 8 is a graph showing the transformation efficiency of competent *E. coli* DH10B that had been lyophilized in glass vials which were vacuum sealed, and stored at -20°C, where the cryoprotectant used was either trehalose or sucrose.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

DNA Molecule. Any DNA molecule, of any size, from any source, including DNA from viral, prokaryotic, and eukaryotic organisms. The DNA molecule may be in any form, including, but not limited to, linear or circular, and single or double stranded. Non-limiting examples of DNA molecules include plasmids, vectors, and expression vectors.

Cloning vector. A plasmid, phage DNA, a cosmid, or other DNA molecule which is able to replicate autonomously in a host cell, and which is

characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (*i.e.*, operably linked to) certain control sequences such as promoter sequences.

Stably stored. Within the meaning of the present invention, competent cells which can be "stably stored" are able to withstand storage for extended periods of time at a suitable temperature, without appreciably losing their transformation efficiency. By the term "without appreciably losing their transformation efficiency" is meant that the competent cells maintain about 40% to 100% preferably 60% to 100%, more preferably 70% to 100%, and most preferably about 80% to 100% of their original transformation efficiency (just after lyophilization) during a storage period of 30 days, preferably 60 days, more preferably 90 days, and most preferably 120 days, at a temperature of -20°C. Suitable storage temperatures vary from about room temperature to about -180°C. Preferably, the storage temperature ranges from about 4°C to about -80°C, more preferably from about -20°C to about -80°C. In a preferred aspect of the invention, the cells are stored at about -20°C. The storage period or time may range from about 0 days to about 150 days, preferably from about 240 days to about 365 days, and more preferably from about 365 days to about 450 days, although longer storage times may be used at temperatures of about -20°C and below.

Competent cells. Cells having the ability to take up and establish an exogenous DNA molecule.

Substantially pure. As used herein means that the desired purified protein is free from contaminating cellular components which would be associated with the protein in nature. "Substantially pure" does not indicate that the protein must be completely free of all contaminants.

The method of the invention provides for the production of cells which are competent for transformation and which may be stably stored for extended periods at various temperatures. Both gram negative and gram positive prokaryotic cells (e.g., bacteria) can be used in accordance with the invention. Examples of suitable prokaryotic cells include, but are not limited to, *Escherichia sp., Klebsiella sp., Salmonella sp., Bacillus sp., Streptomyces sp., Streptococcus sp., Shigella sp., Staphylococcus sp.*, and *Pseudomonas sp.* Non-limiting examples of species within each aforementioned genus that can be used in accordance with the invention include *Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis, Salmonella typhimurium, Streptomyces aureus, Streptococcus mutans, Streptococcus pneumoniae*, and *Pseudomonas syringae*.

In a preferred embodiment, the cells which are rendered competent by the claimed method are *Escherichia*, most preferably *E. coli*. Non-limiting examples of *E. coli* strains which can be made competent by the claimed methods include DH5, DH5α, DH10, DH10B, HB101, RR1, JV30, DH11S, DM1, DH10B/p3, DH5αMCR, DH5α5'IQ, DH5α5', SCS1, Stab2, DH12S, DH5α-E, DH10BAC, XL1-Blue MRF, XL2-Blue MRF, XL1-Blue MR, SURE Strain, SURE 2 Strain, XL1-Blue, XL2-Blue, AG1, JM101, JM109, JM110/SCS110, NM522, TOPP Strains, ABLE Strains, XL1-Red, BL21 Strains, TK B1 Strain, and derivatives thereof.

According to the method of the invention, the cells to be made competent are grown in a growth conducive medium. Any medium capable of supporting growth of the cells to be made competent can be used. Examples of such media include but are not limited to Luria Broth, Luria Broth supplemented with 20 mM $MgCl_2$, 0.001% thiamine, and 0.2% glucose; SOB medium containing 0.001%

PPG (recipe given below); and 15/10 medium (recipe given below). Other suitable media will be readily recognized by one of skill in the art.

The incubation temperatures for growing the cells may vary from about 10° to about 42°C. Preferably, the temperatures range from about 12° to about 37°C, more preferably from about 15°C to about 32°C, and most preferably from about 20° to about 25°C. In a preferred aspect of the invention, the cells are grown at about 23°C.

As one of ordinary skill in the art will understand, growth conditions and culture age can affect both the viability and the transformation efficiency of cells following cryopreservation. Cells grown in shake flask culture are generally more resistant to the stress of freeze-drying than are static broth cultures. Furthermore, the age of a culture also effects the ability of the culture to survive freeze-drying. Generally, cells harvested in late log or early stationary growth exhibit the greatest resistance to freeze-drying.

Thus, in accordance with the present invention, the cells are grown in shake flasks, although other means of growth may be used including fermentators. Shake flasks used in the invention can be of any size and any type. Preferably, baffled 2.8 L liter shake flasks are used for this processing. Incubation times will vary according to the conditions used (temperature, medium, aeration, etc.) and the cell type. Aeration in flasks varies according to the rotation per minute (rpm) used, with higher rpms resulting in higher aeration. Flasks are typically shaken at 100-500 rpms, preferably 200-400 rpms, and most preferably 200-300 rpms, although one of ordinary skill in the art may determine other preferred ranges. Cells are typically grown for a time and under conditions sufficient to reach an optical density (OD) at 550 nm between 0.1 to 2.0. Preferably, the OD ranges from 0.1 to 1.0, more preferably from 0.3 to 0.8, more preferably from 0.5 to 0.8, even more prefcrably from 0.5 to 0.7, still more preferably from 0.6 to 0.8, and most preferably from 0.66 to 0.75.

After the cells have reached the desired OD, the cells may be collected for further processing. In accordance with the invention, if the desired OD is not

reached or is exceeded, the cells can again be reinoculated and the growth process repeated until a culture of sufficient optical density is obtained.

After the cells are collected (by centrifugation, filtration, *etc.*), they may optionally be chilled (*e.g.*, 0° to 4°C for 5 minutes to 2 hours). Collection of the cells may be accomplished by centrifuging the cells to obtain a cell pellet. Collection may also be accomplished by concentrating the cells and then centrifuging the concentrated cultures to obtain a cell pellet. Methods of concentrating the cells include, but are not limited to, dewatering the culture, filtering, or subjecting the culture to size exclusion chromatography, *e.g.* using Centricon™ columns (Amicon Corp., Lexington, MA).

After the cells are collected, the cells are resuspended in a competence buffer. A competence buffer is any solution that enables cells to take up and establish exogenous DNA. Non-limiting examples of competence buffers include 50 mM $CaCl_2$, 10 mM Tris/HCl (Maniatis, T. *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY (1989)); 0.1 M MOPS (pH 6.5), 50 mM $CaCl_2$, 10 mM $RbCl_2$, 23% V/V DMSO; CCMB80 buffer (10 mM potassium acetate pH 7.0, 80 mM $CaCl_2 \cdot H_2O$, 20 mM $MnCl_2 \cdot 4H_2O$, 10 mM $MgCl_2 \cdot 6H_2O$, 10% glycerol adjusted to pH 6.4 with 0.1 N HCl); TFB buffer (Liu *et al.*, *Biotechniques* 8(1):23-25 (1990)); and χ1776 buffer (Maniatis, T. *et al.*, Molecular Cloning: A Laboratory Manual (2nd ed., Cold Spring Harbor, NY (1989)). Other suitable buffers are disclosed in Tang *et al.*, *Nucl. Acids Res. 22(14)*:2857-2858 (1994); Chung *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 86*:2172-2175 (1989); M. Dagert and S.D. Ehrlich, *Gene 6*: 23-28 (1974); Kushner, S.R., *In:* Genetic Engineering (H.W. Boyer and S. Nicosia, eds.) pp. 17-23, Elsevier/North Holland, Amsterdam (1978); Mandel and Higa, *J. Mol. Biol. 53*:159-162 (1970); U.S. Patent No. 4,981,797 by Jessee; and Hanahan, D., *J. Mol. Biol. 166*:557-580 (1983).

The cells suspended in the competence buffer are incubated for a sufficient time and at a temperature sufficient to make the cells competent to DNA uptake. Preferably, the cells are incubated at low temperature (0 to 4°C) for 0 to 3 hours,

more preferably 5 min. to 1 hr., and most preferably 5 min. to 30 min. After the cells have been made competent, a cryoprotectant may be added directly to the cell suspension. Preferably, the cells are collected and then resuspended in a cryoprotectant. The concentration of the cryoprotectant will vary depending on the cell type, buffers used, the type of cryoprotectant and other factors. Optimal conditions can be determined by one skilled in the art without undue experimentation. Cryoprotectants provide protection of the cells during the freezing process by depressing the freezing point, minimizing the effect of solution changes external to the cell, penetrating the cell to protect against solute concentration effects, and/or shifting the optimum cooling rate to lower values (F. P. Simione, *Journal of Parenteral Science & Technology, 46*(6):226-232 (1992)). Of course, the cryoprotectant must not be toxic to the cells. Further information on the preservation of living cells by freeze drying may be found in Simione, 1992.

Any cryoprotectant and combination thereof may be used in accordance with the invention. As will be apparent, the type and amount used may vary depending on the cell type and conditions used. Cryoprotectants that can be used in the present invention include, but are not limited to, carbohydrates and carbohydrate derivatives such as trehalose, sucrose, lactose, maltose, mannitol, galactose, ribose, fructose, xylose, mannose, dextrose, glucose, and sorbitol, and polymers such as polyethyleneamine, polyvinylpyrrolidone (PVP), ficoll, *etc.* Other cryoprotectants which can be used in accordance with the invention, such as acacia gum, albumin, gelatin, and sugar alcohols, will be readily recognized by one skilled in the art.

After the cells have been mixed with the cryoprotectant, the cell suspension may be aliquoted into containers to be used for lyophilization and storage, such as chilled cryovials, *e.g.*, NUNC tubes (Gibco BRL, Gaithersburg, MD, Cat. No. 366656), or glass vials (Wheaton, Millville, N.J.). Prior to lyophilization, the cells are frozen at about -20°C to about -180°C, preferably at about -80°C to about -180°C, most preferably about -80°C.

Methods of freezing a sample to a temperature from about -80° to about -180°C are well known in the art. These include overnight storage (about 16 hours) of the vials which contain the cells in a -80°C freezer, or immersion of the vials which contain the cells in dry ice, or in a low temperature bath, such as dry ice ethanol, or in a bath containing liquid nitrogen. Other such systems are disclosed in *The Chemist's Companion: A Handbook of Practical Data, Techniques, and References*, Gordon, A.J., *et al.*, eds., John Wiley and Sons, NY (1972).

The cells are then lyophilized by techniques which are well known in the art. Lyophilization is a process by which ice and/or moisture is removed from frozen cells by sublimation under vacuum at low, subzero temperatures (*e.g.*, -40° to -50°C). Any residual moisture associated with the "dried" preparation is then removed by gradually raising the temperature, resulting in evaporation. Thus, according to the invention, lyophilization comprises subjecting frozen cells to a vacuum under conditions sufficient to substantially remove moisture and/or ice from said cells (also referred to herein as substantially dried cells). The substantially dried cells may then be stored at various temperatures (room temperature to about -180°C, preferably about 4°C to about -80°C, more preferably about -20°C to about -80°C, and most preferably about -20°C).

One such process for lyophilizing cells comprises the steps of

(a) loading a container containing frozen cells into a lyophilizer, the lyophilizer having a temperature of about -40° to about -50°C;

(b) subjecting the cells to a vacuum; and

(c) substantially drying the cells.

Preferably, the vacuum is less than about 100 μm, and the cells are dried by:

(i) holding the temperature of the chamber at about -45°C for about 2 hours; and

(ii) increasing the temperature of the chamber from about -45°C to about 10°C at the rate of about 0.1° to 1.0°C/hr (preferably, 0.5° to 0.8°C/hr, and most preferably 0.6° to 0.8°C/hr).

The cell container may then be sealed and stored for extended time at various temperatures.

The viable cell count of competent cells produced by the method of the invention will remain at greater than about 1×10^7 cells/ml, preferably greater than about 1×10^8 cells/ml, and more preferably greater than about 1×10^9 cells/ml when stored at -20°C for any time period from about 0 days to about 450 days, preferably from about 240 days to about 365 days, and more preferably from about 365 days to about 450 days. These cells will retain a transformation efficiency of at least about 1×10^5, preferably at least about 1×10^6, more preferably at least about 1×10^7, still more preferably at least about 1×10^8 and most preferably at least about 1×10^9 transformants per microgram of DNA (T/μg). Suitable storage temperatures vary from about room temperature to about -180°C. Preferably, the storage temperature ranges from about 4°C to about -80°C, more preferably from about -20°C to about -80°C. In a preferred aspect of the invention, the cells are stored at about -20°C. The storage period or time may range from about 0 days to about 45 days, preferably from about 0 days to about 90 days, still more preferably from about 0 days to about 150 days, yet more preferably from about 240 days to about 365 days, and still more preferably from about 365 days to about 450 days, although longer storage times may be used at temperatures of about -20°C and below. Competent cells produced by the method of the invention may be stored at -20°C for at least one year while retaining substantially their transformation efficiency. Substantial retention of transformation efficiency means that after lyophilization, the cells will have a transformation efficiency after storage that is about 40% to 100%, preferably at about 60% to 100%, more preferably about 70% to 100% and most preferably about 80% to 100% of the transformation efficiency of the cells immediately after lyophilization.

The invention also pertains to transforming a lyophilized competent cell produced according to the method of invention. Transforming said lyophilized competent cells comprises obtaining a lyophilized competent cell, mixing said cell with a DNA molecule, and incubating said mixture under conditions sufficient to transform said cell with said DNA molecule. According to this aspect of the invention, the lyophilized competent cell may be any gram positive or gram negative bacteria including, but not limited to, *Escherichia, Klebsiella, Salmonella, Bacillus, Streptomyces, Streptococcus,* and *Pseudomonas.* Preferably, gram negative prokaryotic cells are transformed according to the method of the invention, more preferably *Escherichia,* and most preferably *E. coli.* According to the invention, any DNA molecule (*e.g.* vectors, plasmids, phagemids, expression vectors, *etc.*) may be used. Preferably, the cells are mixed with the DNA molecule in the presence of a competence buffer. According to the invention, the competence buffer may be added to the lyophilized competent cells prior to adding the DNA molecule or the DNA molecule and competence buffer may be added simultaneously to the lyophilized competent cells. Although mixing of the lyophilized competent cell with the DNA molecule and a competence buffer is preferred, any solution may be used to rehydrate and mix the competent cells with the DNA molecule. Such solutions include water, saline, or any suitable buffer.

After the cells have been transformed with the DNA molecule of interest, the transformed cells may be grown in a growth conducive medium. Typically, such a growth conducive medium contains an antibiotic to assist in selection of transformed cells. That is, the DNA molecule to be transformed may contain a selective marker (*e.g.* an antibiotic resistance gene), allowing selection of transformed cells when the corresponding antibiotic is used in the medium.

The invention also concerns a method of producing a desired protein by transforming a lyophilized competent cell with a DNA molecule encoding said desired protein. Thus, the invention concerns a method of producing a desired protein comprising obtaining a lyophilized competent cell produced according to

the invention, transforming said cell with a DNA molecule capable of expressing said desired protein, and culturing said transformed cell under conditions sufficient to produce said desired protein. Cells which can be used according to this aspect of the invention including both gram negative and gram positive bacteria, preferably *Escherichia*, and most preferably *E. coli*. In this aspect of the invention, the cells are transformed by mixing the cells with a DNA molecule and incubating the mixture under conditions sufficient to transform said cell with said DNA molecule. This mixing step may be accomplished in any solution. Most preferably, the lyophilized competent cells are rehydrated in a competence buffer prior to adding the DNA molecule, or the lyophilized competent cells are simultaneously mixed with the DNA molecule and the competence buffer. Transformed cells may be selected according to techniques well known in the art including, for example, selection for marker genes on the DNA molecule (*e.g.* antibiotic resistance genes). After the transformed cell has been selected, the cell may then be cultured according to well known techniques in a growth conducive medium. Upon culturing the cell under appropriate conditions, the cell is capable of producing the desired protein. The desired protein may then be isolated, and a substantially pure protein obtained by well known protein purification techniques.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended to be limiting.

Examples

Example 1

A seed stock of *E. coli* DH5α cells (Gibco BRL, Gaithersburg, MD) was prepared as follows: DH5α cells were streaked on an LB plate (32 g Gibco BRL LB agar (Life Technologies, Inc., Gaithersburg, MD) per liter distilled water) and

the plate was incubated for 36 hours at 23°C. Several colonies were picked into a 500 ml non-baffled shake flask containing 25 ml SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$).

The flask was shaken at 23°C, 275 rpm, for several hours and the optical density at 550 nm is followed. When the optical density had reached 0.5, 10 ml of the cells were mixed with 10 ml of SOB:glycerol 60:40 (60 ml SOB, 40 ml glycerol, (Gibco BRL) in a 50 ml conical tube. The cells were mixed using a vortex mixer and were allowed to remain for 10 min. on ice. 1 ml aliquots were dispensed into NUNC cryovials (Catalog No. 366656) (Life Technologies, Inc., Gaithersburg, MD) and were frozen in a dry ice ethanol bath for at least 5 minutes. The seeds were stored at -80°C.

A seed of DH5α cells stored at -80°C was thawed on ice for 10 min. 0.450 ml of the thawed seed was inoculated into 1500 ml of SOB medium containing 0.001% PPG in a 2.8 L Fernbach flask. The flask was shaken at 23°C (275 rpm). After approximately 20 hours, the culture had reached an optical density at 550 nm of 0.66. The cells were chilled on ice for 15 min. and collected by centrifugation using Corning 250 ml bottles with 250 ml of cell culture/bottle. The cells were centrifuged in a GS3 rotor at 4000 rpm 4°C for 10 minutes in a Sorvall RC2B centrifuge.

Each cell pellet was resuspended in 75 ml of cold CCMB80 buffer (10 mM potassium acetate pH 7.0, 80 mM $CaCl_2 \cdot H_2O$, 20 mM $MnCl_2 \cdot 4H_2O$, 10 mM $MgCl_2 \cdot 6H_2O$, 10% glycerol adjusted to pH 6.4 with 0.1 N HCl). The cells were kept on ice for 20 min. The cell pellet was collected at 4°C by centrifugation and each cell pellet was resuspended in 16 ml of either 9% aqueous trehalose (Quadrant, Cambridge England) or 12% aqueous sucrose. (Life Technologies Inc., Gaithersburg, MD). 250 µl of the cell suspension was aliquoted into chilled 1.0 ml NUNC cryovials (Life Technologies, Inc., Gaithersburg, MD, Catalogue No. 366656). The caps were placed loosely on top of the vials and the vials were placed in a -80°C freezer for 16 hours.

Alternatively, the cells were vialed in chilled 5 ml glass vials (Wheaton, Millville, N.J., Catalogue No. 223712). A rubber stopper was placed loosely on top of the vial, and the vial was placed in a -80°C temperature for 16 hours.

After overnight storage in the -80°C freezer, the vials were placed in a Hull lyophilizer and were subjected to vacuum drying according to the following protocol:

(a) Adjust shelf temperature to -45°C.

(b) Load frozen DH5α samples evenly on each shelf.

(c) Close the chamber and turn on the vacuum system. When the vacuum is less than 100 m., start the drying program composed of the following 3 segments:

i) Hold the shelf temperature at -45°C for 2 hours.

ii) Increase the shelf temperature from -45°C to 10°C at the rate of about 0.7°C/hour (55°C in 72 hours).

(d) As soon as the shelf temperature reaches 10°C, the cells are moved to a 4°C cold room.

(e) Store the vials at -20°C.

When the lyophilization cycle was complete, the cells were removed from the lyophilizer and taken to a 4°C cold room where the caps were tightened. The cells were then placed in a foil pouch containing desiccant and the pouches were placed in a -20°C freezer for storage.

To assay the cells for the transformation efficiency and viable cell count, the vials were removed from the -20°C freezer and placed on wet ice for 6 minutes. The cells were rehydrated in 400 µl of a 1:1 mixture of CCMB80 buffer without glycerol (10 mM potassium acetate pH 7.0, 80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MnCl_2 \cdot 4H_2O$, 10 mM $MgCl_2 \cdot 6H_2O$, adjusted to pH 6.4 with 0.1 N HCl) and FSB buffer (10 mM potassium acetate pH 7.0, 100 mM KCl, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM hexaaminecobalt(III) chloride, 10% glycerol, 5% aqueous sucrose adjusted to pH 6.4 with 0.1 N HCl). 100 µl of the cells were

removed from the vial and placed in a chilled Falcon™ 2059 tube (Becton Dickenson) for assay with 50 pg of pUC19 plasmid DNA. The viable cell count of the resuspended cells was also determined by standard dilution using 0.85% NaCl. The cells were then reassayed for transformation efficiency and viable cell count after storage at -20°C. The results of one such stability study is presented in Figures 1, 2 and 3.

Figure 1 indicates that DH5α cells lyophilized in NUNC cryovials using either sucrose or trehalose as the cryoprotectant retained a transformation efficiency of $>1.0 \times 10^7$ T/ug when stored for at least 12 months at about -20°C. Figure 2 indicates that the viable cell count of the cells stored in NUNC cryovials at about -20°C remained at approximately 1.0×10^9 cells/ml for at least 12 months. Figure 3 indicates that the cells lyophilized using trehalose in glass vials which are vacuum sealed retain a transformation efficiency of $>1.0 \times 10^8$ T/μg after storage at -20°C for 12 months.

Example 2

The following example was carried out essentially as Example 1 with the following exceptions. The DH5α seed stored at -80°C was thawed and 600 μl of the seed was inoculated into 1.7 L of SOB medium containing 0.001% PPG in a 2.8 L Fernbach flask. The flask was shaken for 18 hours at 23°C, 275 rpm. When the optical density at 550 nm had reached 0.194, 175 ml of the culture, was inoculated into 1.7 L of SOB medium containing 0.001% PPG in a 2.8 L Fernbach flask. The flask was shaken for approximately 4 hours at 23°C, 275 rpm. When the optical density at 550 nm had reached 0.09, 2.7 ml of the culture was inoculated into 2 Fernbach flasks each containing 1.7 L SOB medium and 0.001% PPG. The flasks were shaken at 23°C, 275 rpm, for approximately 22 hours. When the optical density at 550 nm had reached between 0.648 and 0.722, the cultures were harvested and processed as in Example 1, with the exception that the cells were not chilled prior to collection by centrifugation. 200 ml of cells

were then centrifuged at about 4°C as described in Example 1 and the cell pellet was resuspended in 60 ml cold CCMB80 buffer. The cells were placed on ice for 20 minutes and again collected by centrifugation at about 4°C. The cell pellet was resuspended in 25.6 ml of cold 12% aqueous sucrose and the cells were placed on ice for 2 hours. 250 µl of the cells were vialed after a 2 hour incubation on ice into chilled NUNC cryovials. The cells were frozen by placing the vials in a -80°C freezer for 16 hours or were frozen by immersion in a liquid nitrogen bath. The cells were lyophilized as described in Example 1.

The results are presented in Figures 4A, 4B, and 5. As seen in Figures 4A and 4B, the cells lyophilized in sucrose could be stored at -20°C for at least 5 months without substantial loss of transformation efficiency. The transformation efficiency of the samples was at least 1 X 10^7 T/ug. Samples frozen in either liquid nitrogen (Figure 4B) or by storage at -80°C for 16 hours (Figure 4A) prior to lyophilization retained a transformation efficiency of >1.0 x 10^7 T/µg. As seen in Figure 5, the lyophilized DH5α cells could be stored at -20°C without significant loss of cell viability. Freezing in either liquid nitrogen or by storage at -80°C for 16 hours prior to lyophilization resulted in stable storage of the cells. All samples had remained on ice for at least 2 hours before vialing. Therefore this data indicates that the cells may remain on ice in the cryoprotectant for at least 2 hours prior to freezing and lyophilization while substantially retaining transformation efficiency and cell viability.

Example 3

The following example was carried out essentially as in Example 2 with the following exceptions. Two DH5α seeds were thawed and 800 µl was inoculated into two 2.8 L Fernbach flasks, each containing 1.7 L of SOB medium and 0.001% PPG. The flasks were shaken at 23°C, 275 rpm, for approximately 19 hours. The optical density of the two flasks were 0.3918 and 0.3578, respectively. The cells were collected by centrifugation at 4°C. Each cell pellet

was resuspended in 10 ml of room temperature SOB medium containing 0.001% PPG and were pooled (total of 120 ml). A 1:100 dilution of the cells had an optical density of 0.0754, indicating that the cell density was 7.54. Twenty ml of the cells were inoculated into 6 Fernbach flasks, each containing 1.7 L SOB medium and 0.001% PPG. The flasks were shaken at 23°C, 275 rpm, for 6.5 hours, at which time the optical densities of the flasks were 0.705, 0.783, 0.701, 0.749, 0.704, and 0.702. The flasks were chilled on ice for 15 minutes. Ten liters of the cells were concentrated in the 4°C cold room by dewatering to 3 L in the following manner.

The peristaltic pump used was a Masterflex model 7529-30 (Cole Palmer) and the column used was a Microgon column # M22M-300-01N. The column was connected to the pump and rinsed once with 2 liters of autoclaved deionized water. The pump was turned on and 2 liters of 0.37% bleach was circulated through the system for 20 minutes. The system was then rinsed with 10 liters of autoclaved deionized water. 10 liters of the cell suspension was poured into the reservoir. The cell suspension was then circulated through the system with the pump speed set at 3.5 to reduce the volume to 3 liters. The process required approximately 25 minutes (approximately 280 ml/minute). The 3 liters of concentrated cell suspension was recovered from the reservoir by pumping the cell suspension out of the reservoir.

Six bottles each containing 250 ml of the concentrated cell suspension were centrifuged in a GS3 rotor at 4000 rpm for 10 minutes at 4°C. Each cell pellet was resuspended in 250 ml of cold CCMB80 buffer. The cells remained on ice for 20 minutes, and the cells were then collected by centrifugation at 4°C. Each cell pellet was resuspended in 53 ml of cold 9% aqueous trehalose. The cells were placed on ice for 1 hour, and 250 µl of the cells were vialed in 1.0 ml cryovials (Wheaton, Millville, N.J.). The vials were placed in a -80°C freezer and remained for 16 hours before lyophilization. The cells were lyophilized in a Tri-Philizer™ model TRI585 lyophilizer (FTS Systems Inc.) with the temperature being increased at a rate of 0.6°C/hr.

The results are presented in Figure 6 and 7. Figure 6 indicates that the cells can be stored at -20°C for at least 4 months and maintain a transformation efficiency greater than 1 x 10^7 T/ug. Figure 7 indicates that the cell viability remains at greater than 1 x 10^9 cells/ml after storage for 4 months at -20°C.

Example 4

This Example was performed essentially as Example 1 with the following exceptions. *E. coli* strain DH10B was streaked from a master seed stored at -80°C onto an LB plate containing 100 µg/ml streptomycin. The plate was incubated for 24 hours at 23°C. A single colony was picked from the plate and inoculated into a flask containing 250 ml of 15/10 medium (1.0% Bacto tryptone, 1.5% Bacto yeast extract, 10 mM NaCl, 2 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 0.001% polypropylene glycol (PPG)). The flask was shaken at 23°C, 260 rpm in a New Brunswick Psycrotherm™ shaker for 16 hours. Six ml of the culture was inoculated into a 2.8 L Fernbach flask containing 1.25 L of 15/10 medium, and the flask was shaken at 23°C, 260 rpm. The optical density at 550 nm was initially 0.1. The flask was shaken until the optical density was 0.7, and the cells were then collected by centrifugation at 4°C. The cells were processed as in Example 1. After resuspension in the cryoprotectant, 1 ml of the cell suspension was aliquoted into sterile 5 ml glass vials (Wheaton) and the cells were frozen for 5 minutes in a dry ice ethanol bath. Rubber stoppers were fitted loosely on the vials and the vials were placed in the lyophilizer. The cells were lyophilized according to the program described in Example 1 except that the vials were sealed under vacuum.

After lyophilization, the vials were removed from the lyophilizer, and were stored at -20°C in foil pouches containing desiccant. At intervals, vials were removed from the freezer and were assayed for transformation efficiency essentially as in Example 1, with the exception that the cells were resuspended in 2 ml of CCMB80 buffer without glycerol. The results are presented in Figure 8.

The data indicate that *E. coli* strain DH10B can be lyophilized and stored at -20°C for at least 6 months while substantially retaining their transformation efficiency. Samples retain a transformation efficiency of $>1 \times 10^8$ T/μg after extended storage at -20°C.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and Examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

What Is Claimed Is:

1. A method for producing cells which are competent for transformation, said method comprising

(a) growing said cells in a growth conducive medium;

(b) rendering said cells competent; and

(c) lyophilizing said competent cells.

2. The method of claim 1, wherein said cells are grown at about 10°C to about 42°C.

3. The method of claim 1, wherein said cells are grown at about 12°C to 37°C.

4. The method of claim 1, wherein said cells are grown at about 15°C to about 32°C.

5. The method of claim 1, wherein said cells are grown at about 20°C to about 25°C.

6. The method of claim 1, wherein said cells are grown at about 23°C.

7. The method of claim 1, wherein said cells are gram negative prokaryotic cells.

8. The method of claim 7, wherein said cells are selected from the group consisting of *Escherichia, Klebsiella, Salmonella,* and *Pseudomonas.*

9. The method of claim 8, wherein said cells are *Escherichia* cells.

10. The method of claim 9, wherein said cells are *E. coli* cells.

11. The method of claim 10, wherein said *E. coli* cells are selected from the group consisting of DH5, DH5α, DH10, DH10B, HB101, RR1, JV30, DH11S, DM1, DH10B/p3, DH5αMCR, DH5α5'IQ, DH5α5', SCS1, Stab2, DH12S, DH5α-E, DH10BAC, XL1-Blue MRF, XL2-Blue MRF, XL1-Blue MR, SURE Strain, SURE 2 Strain, XL1-Blue, XL2-Blue, AG1, JM101, JM109, JM110/SCS110, NM522, TOPP Strains, ABLE Strains, XL1-Red, BL21 Strains, TK B1 Strain, and derivatives thereof.

12. The method of claim 1 wherein said lyophilizing step comprises

(a) freezing the cells; and

(b) subjecting said frozen cells to a vacuum under conditions sufficient to substantially dry said cells.

13. The method of claim 1, wherein said step of rendering said cells competent comprises suspending the cells in a competence buffer.

14. The method of claim 13, wherein said competence buffer is CCMB80 buffer.

15. The method of claim 1, wherein said method further comprises mixing said cells with a cryoprotectant prior to the lyophilizing step.

16. The method of claim 15, wherein said cryoprotectant is a carbohydrate or derivative thereof.

17. The method of claim 16, wherein said carbohydrate is selected from the group consisting of trehalose, sucrose, sorbitol, glucose, and acacia.

18. The method of claim 16, wherein said carbohydrate is trehalose, sucrose or mixtures thereof.

19. The method of claim 1, wherein said lyophilized cells are stored at a temperature from about room temperature to about -180°C.

20. The method of claim 1, wherein said lyophilized cells are stored at about 4°C to about-80°C.

21. The method of claim 1, wherein said lyophilized cells are stored at about -20°C to about -80°C.

22. The method of claim 1, wherein said lyophilized cells are stored at about -20°C.

23. The method of claim 1, wherein said lyophilized cells have a transformation efficiency of at least about 1 x 10^6 transformants per μg of DNA.

24. The method of claim 1, wherein the transformation efficiency of said lyophilized cells is substantially retained after storage.

25. The method of claim 24, wherein said storage is from 0 to about 450 days.

26. The method of claim 24, wherein said storage is from about 240 days to about 365 days.

27. The method of claim 24, wherein said storage is from about 365 days to about 450 days.

28. Competent cells produced by the method of any one of claims 1, 12, or 15.

29. The competent cells of claim 28, wherein said competent cells are *Escherichia* cells.

30. The competent cells of claim 28, wherein said cells are *E. coli* cells.

31. A method for transforming a lyophilized competent cell comprising

(a) obtaining a lyophilized competent cell produced by the method of any one of claims 1, 12, or 15;

(b) mixing said cell with a DNA molecule; and

(c) incubating said mixture under conditions sufficient to transform said cell with said DNA molecule.

32. The method of claim 31, wherein said cells are *Escherichia* cells.

33. The method of claim 32, wherein said cells are *E. coli* cells.

34. The method of claim 31, wherein said DNA molecule is a vector.

35. The method of claim 31, which further comprises rehydrating said lyophilized cells in a competence buffer prior to said mixing step.

36. The method of claim 31, wherein said mixing step involves mixing said lyophilized cells with a competence buffer and the DNA molecule.

37. Transformed competent cells produced by the method of claim 31.

38. A method of producing a desired protein comprising

(a) obtaining a lyophilized competent cell produced by the method of any one of claims 1, 12 or 15;

(b) transforming said cell with a DNA molecule capable of expressing said desired protein; and

(c) culturing said transformed cell under conditions sufficient to produce said desired protein.

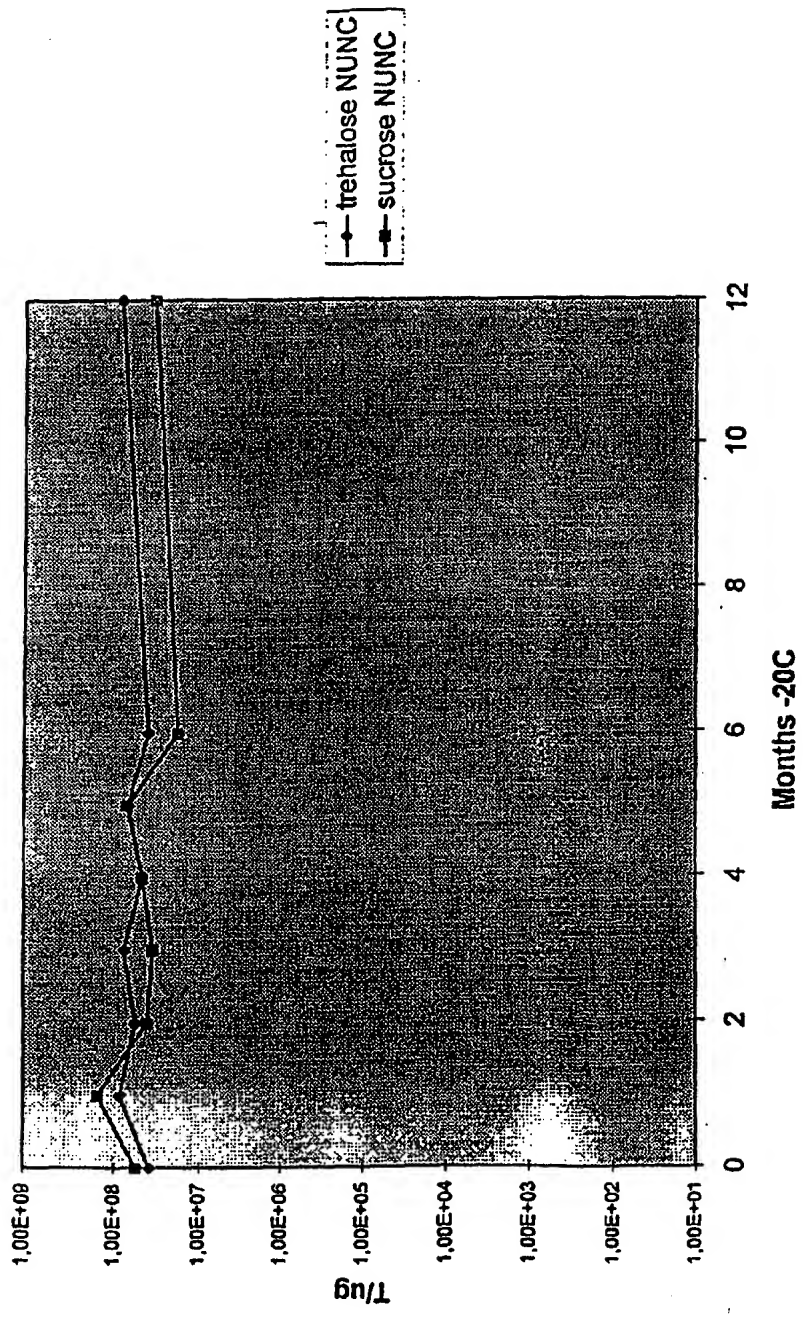
39. The method of claim 38, wherein said cell is an *Escherichia* cell.

40. The method of claim 39, wherein said cell is an *E. coli* cell.

41. The method of claim 38, wherein said DNA molecule is a vector.

42. The method of claim 38, wherein said transforming step comprises mixing said cell with competence buffer and said DNA molecule.

43. A protein produced by the method of claim 36.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sucrose NUNC
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1,00E+04
1,00E+03
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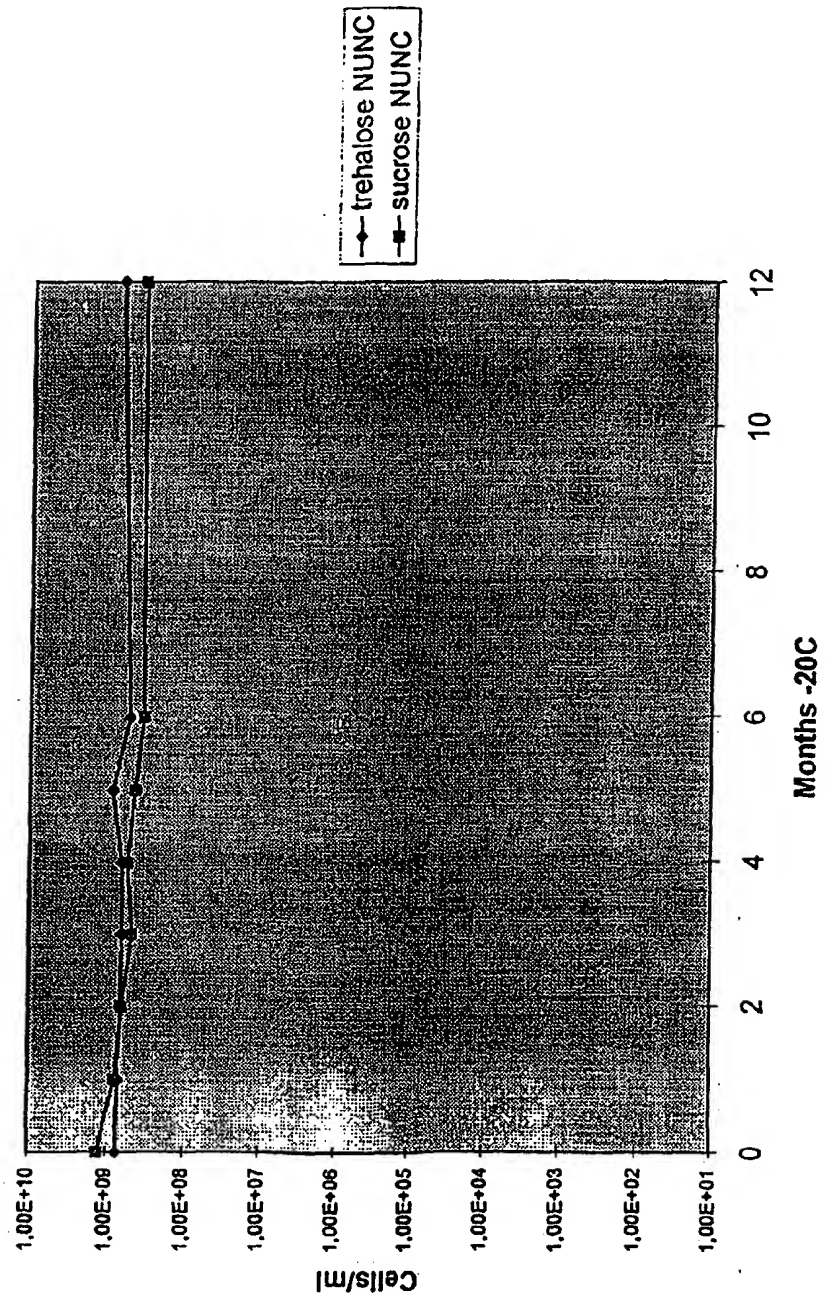
0
2
4
6
8
10
12
Months -20C

Figure 1.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sucrose NUNC
Cells/ml
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1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
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1,00E+01
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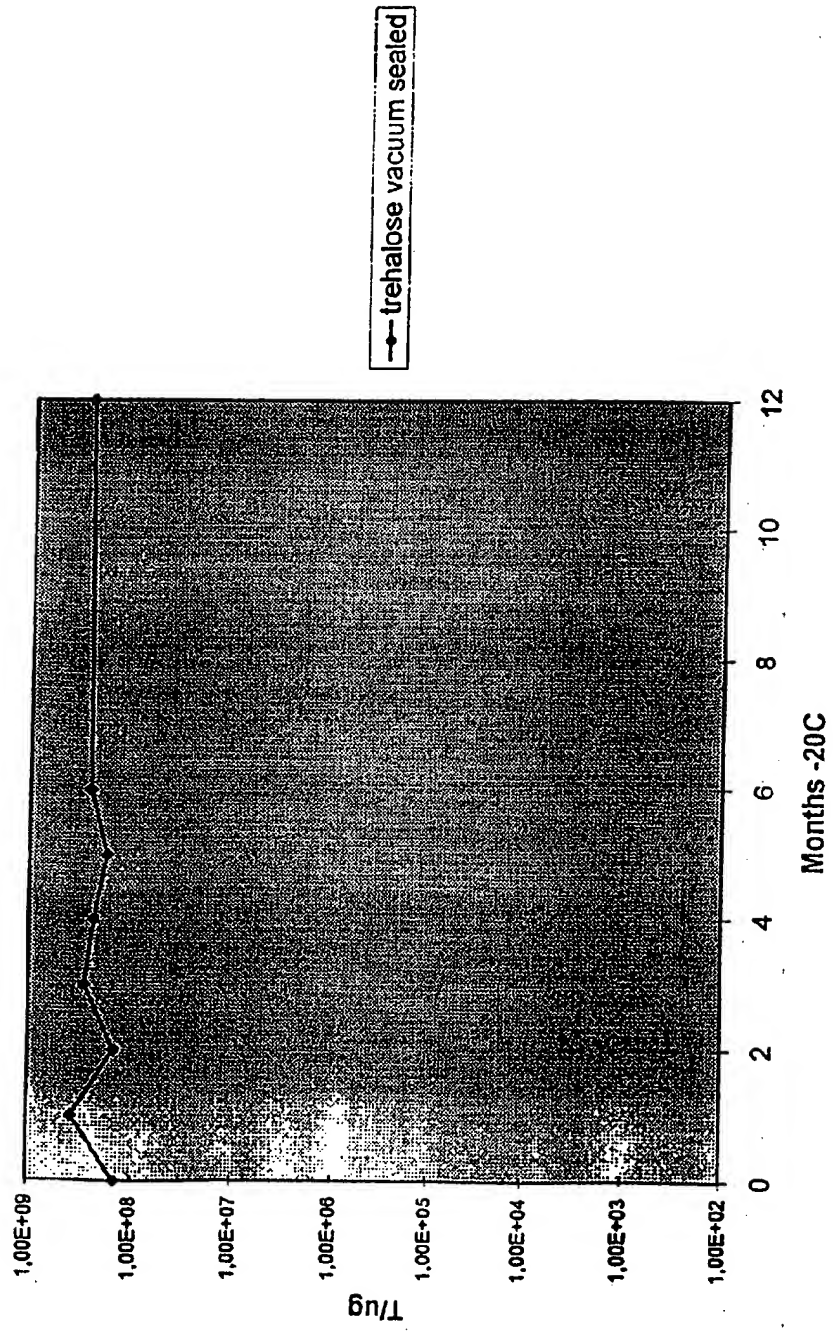
2
4
6
8
10
12
Months -20C

Figure 2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T/ug
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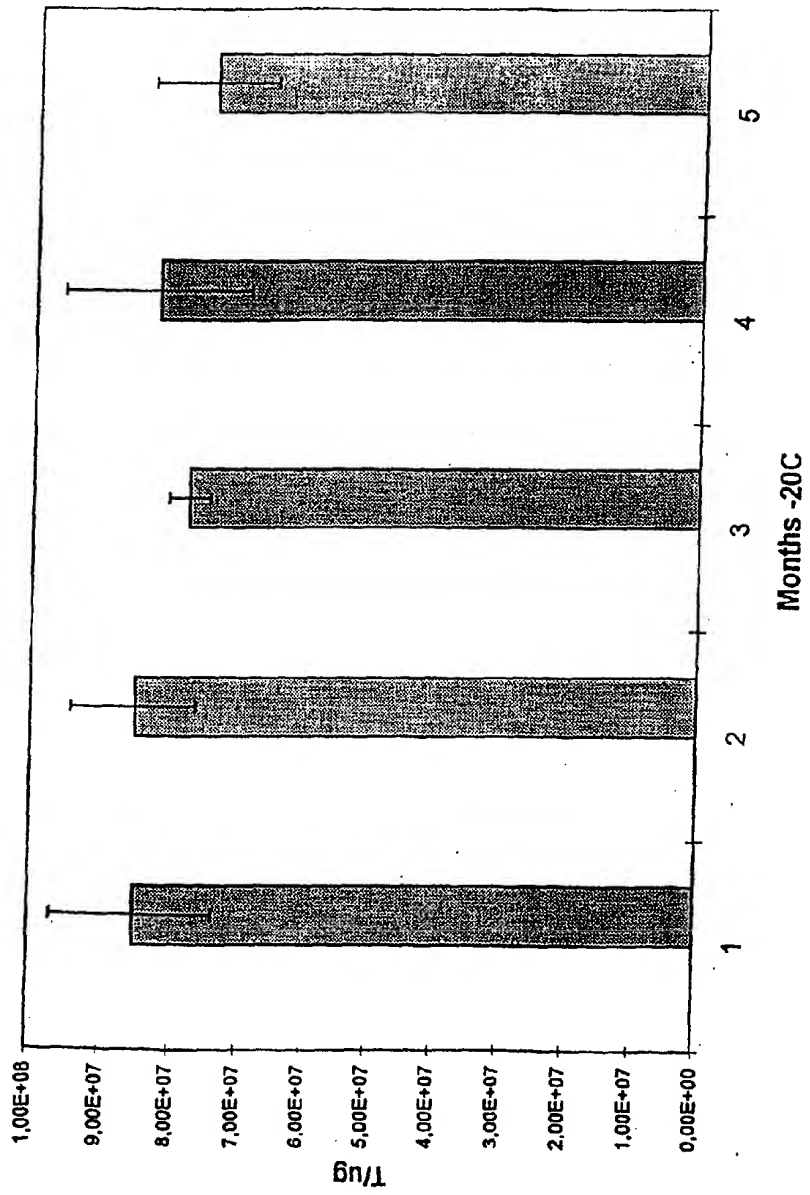
4
6
8
10
12
Months -20C

Figure 3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1
2
3
4
5
Months -20C

Figure 4A

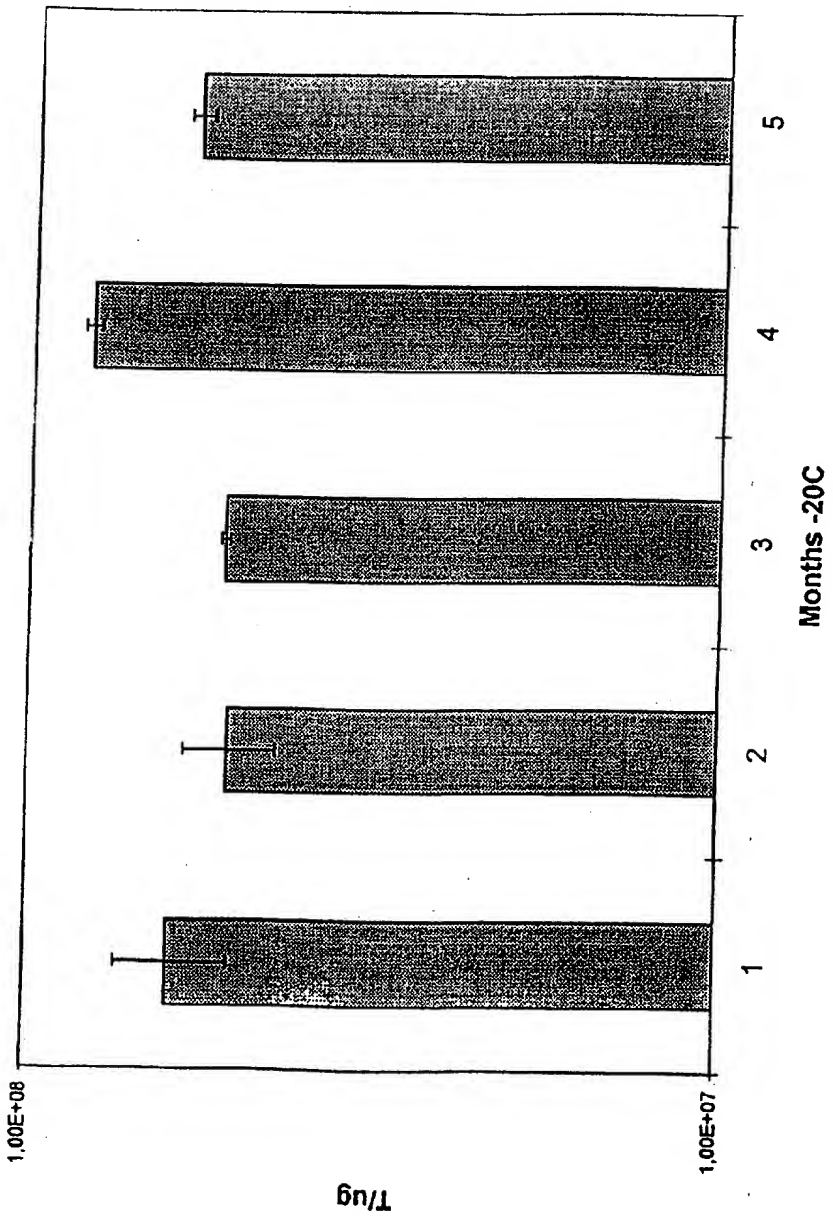
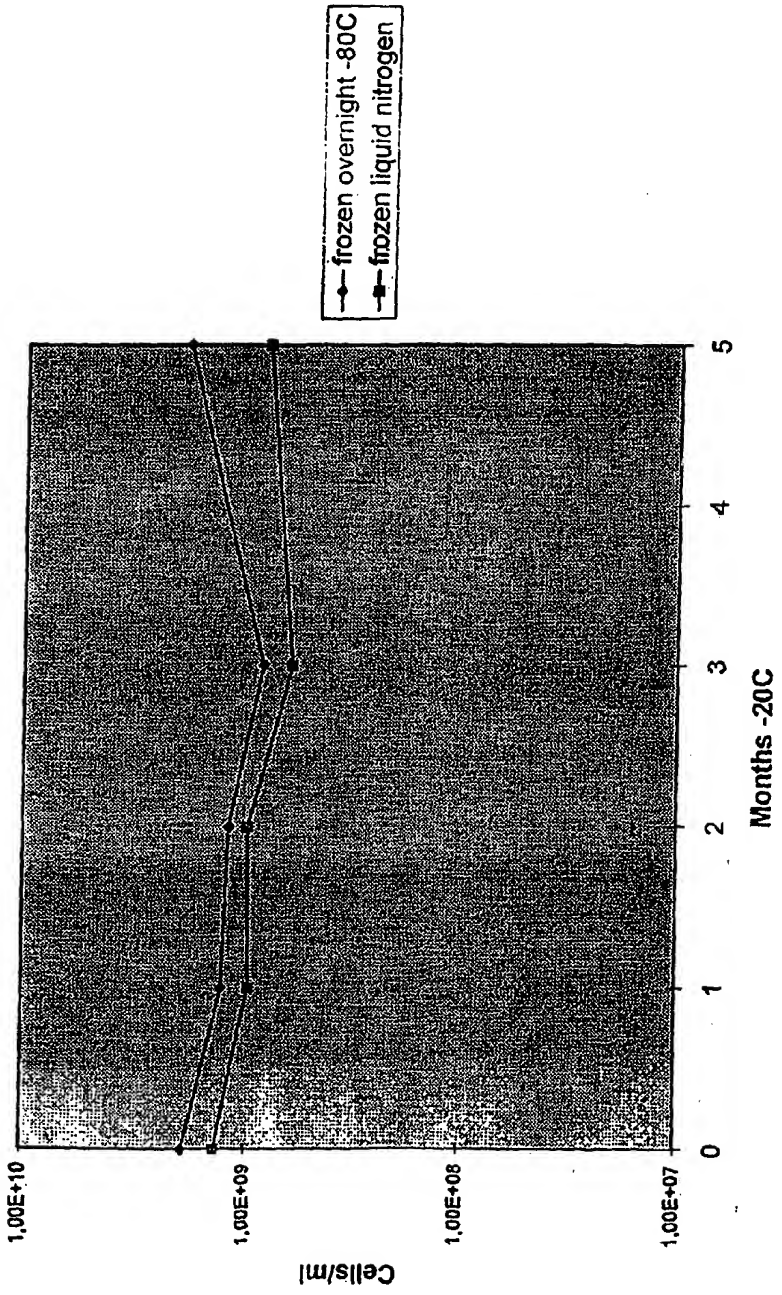


Figure 4B

Figure 5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frozen liquid nitrogen
Cells/ml
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1,00E+08
1,00E+07
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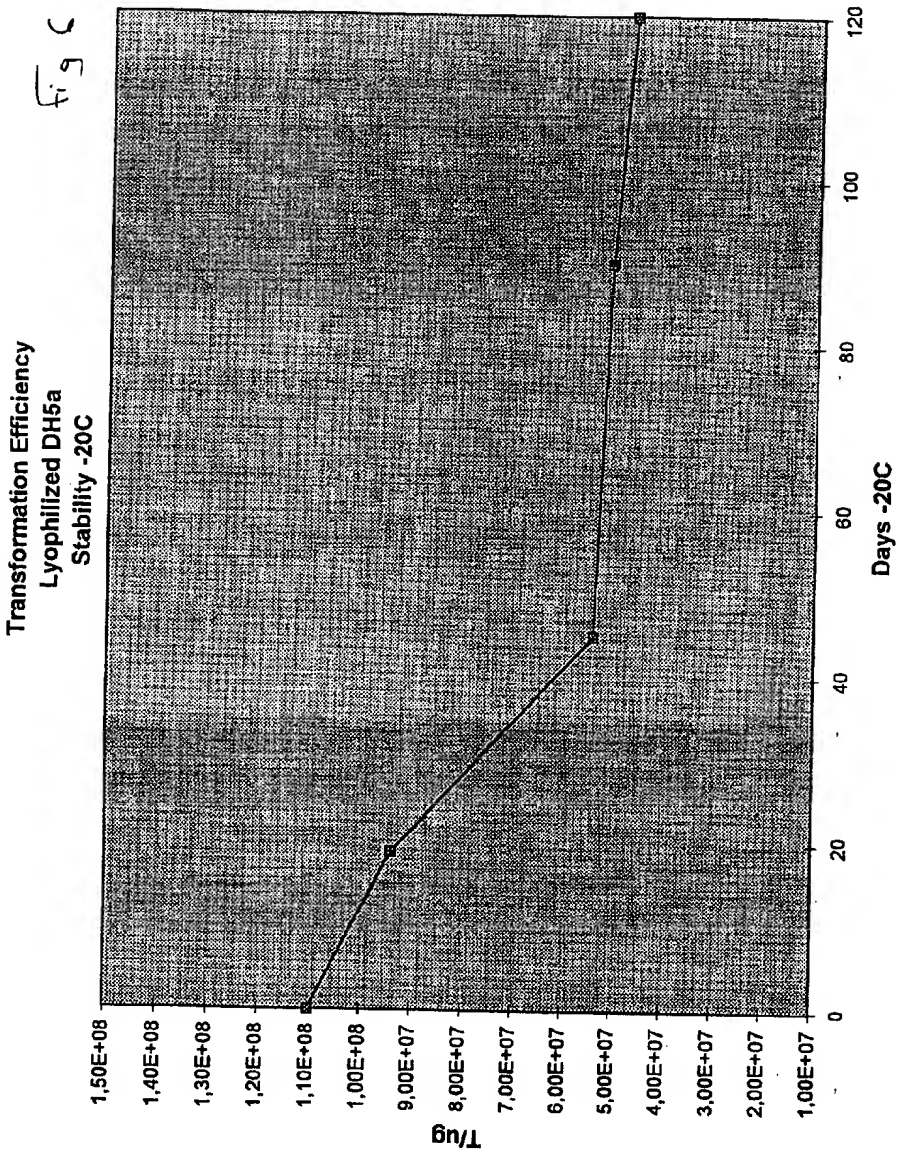
1
2
3
4
5
Months -20C

Fig 6


Transformation Efficiency
Lyophilized DH5a
Stability -20C
T/ug
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1,40E+08
1,30E+08
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9,00E+07
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7,00E+07
6,00E+07
5,00E+07
4,00E+07
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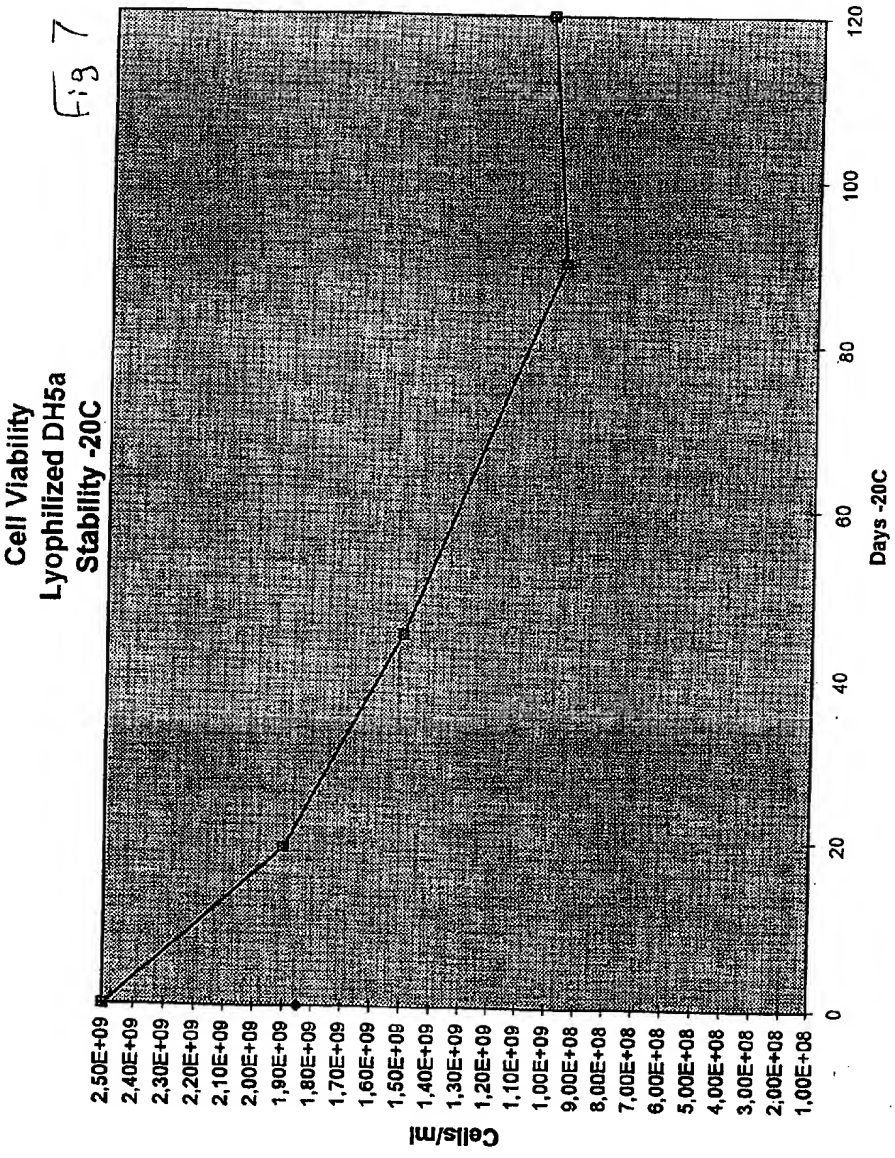
2,00E+07
1,00E+07
0
20
40
60
80
100
120
Days -20C

Fig 7


Cell Viability
Lyophilized DH5a
Stability -20C
Cells/ml
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2,40E+09
2,30E+09
2,20E+09
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2,00E+09
1,90E+09
1,80E+09
1,70E+09
1,60E+09
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4,00E+08
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1,00E+08
0
20
40
60
80
100
120
Days -20C
Page 1

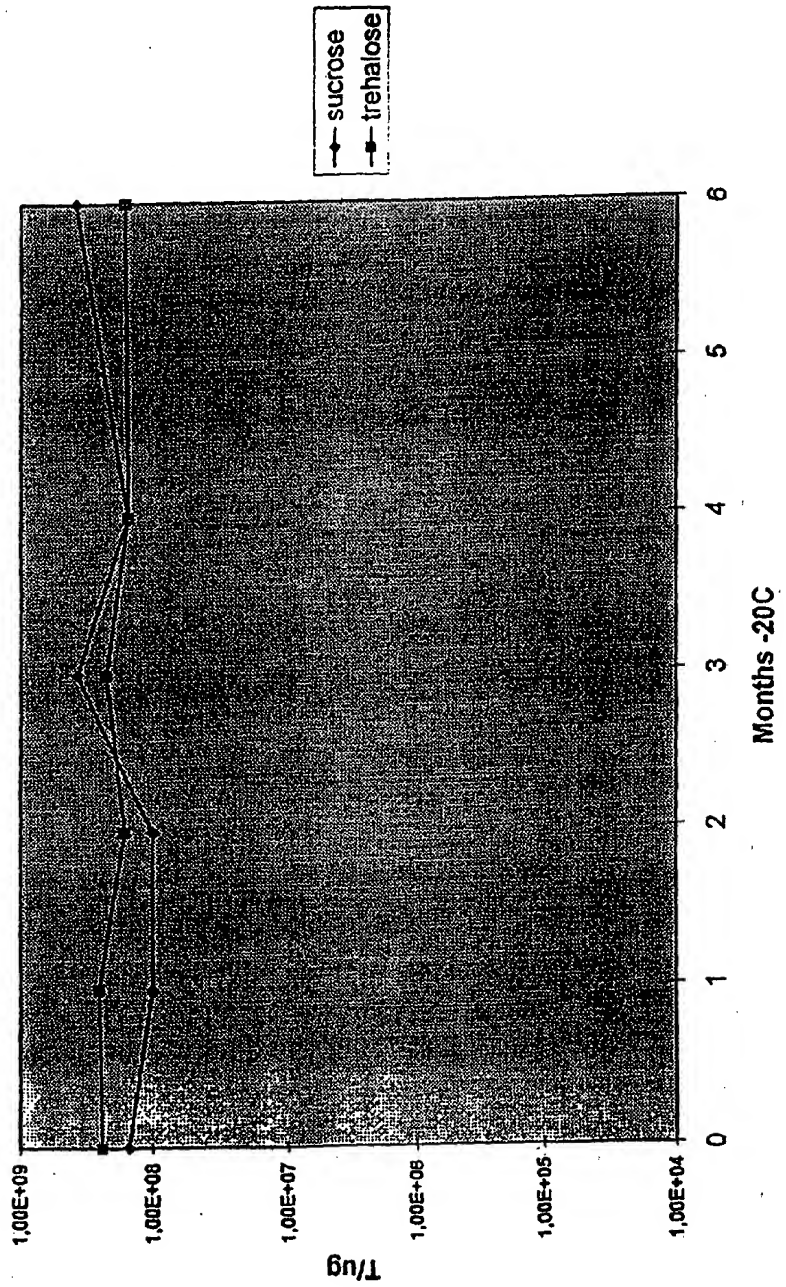
sucrose
trehalose
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
T/ug
0
1
2
3
4
5
6
Months -20C

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02880

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12N 1/21, 15/64; C12P 21/02
US CL : 435/252.33, 172.3; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/252.33, 172.3; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	'1993-1994 Catalogue and Reference Guide', published 1993, by GIBCO BRL, see pages 6-10 and 9-4.	31-43

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 MAY 1998	Date of mailing of the international search report 28 MAY 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAMES KETTER Telephone No. (703) 308-0196

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